Invertase Signal and Mature Sequence Substitutions That Delay Intercompartmental Transport of Active Enzyme

IRENE SCHAUER, SCOTT EMR, COLEMAN GROSS, and RANDY SCHEKMAN
Department of Biochemistry, University of California, Berkeley, California 94720. Dr. Schauer's present address is Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, Colorado 80309. Dr. Emr's present address is Division of Biology, California Institute of Technology, Pasadena, California 91125.

ABSTRACT The role of structural signals in intercompartmental transport has been addressed by the isolation of yeast invertase (*SUC2*) mutations that cause intracellular accumulation of active enzyme. Two mutations that delay transport of core-glycosylated invertase, but not acid phosphatase, have been mapped in the 5′ coding region of *SUC2*. Both mutations reduce specifically the transport of invertase to a compartment, presumably in the Golgi body, where outer chain carbohydrate is added. Subsequent transport to the cell surface is not similarly delayed. One mutation (*SUC2*-s1) converts an ala codon to val at position −1 in the signal peptide; the other (*SUC2*-s2) changes a thr to an ile at position +64 in the mature protein. Mutation s1 results in about a 50-fold reduced rate of invertase transport to the Golgi body which is attributable to defective signal peptide cleavage. While peptide cleavage normally occurs at an ala-ser bond, the s1 mutant form is processed slowly at the adjacent ser-met position giving rise to mature invertase with an N-terminal met residue. s2 mutant invertase is transported about sevenfold more slowly than normal, with no delay in signal peptide cleavage, and no detectable abnormal physical property of the enzyme. This substitution may interfere with the interaction of invertase and a receptor that facilitates transport to the Golgi body.

The compartmentation of eucaryotic cells implies both specific mechanisms for protein localization and identifying signals that are recognized by the localization apparatus. Protein transport must be targeted not only to distinct organelles but to specific subdivisions within such organelles as the mitochondrion, chloroplast, endoplasmic reticulum (ER),[1] Golgi body, and plasma membrane. This process probably involves a number of unique identifying signals of which only a few have been deciphered. Clearly distinct N-terminal signal peptides direct secretory, mitochondrial, and chloroplast precursors to their respective organelles (1–3); hydrophobic membrane anchoring sequences have been recognized in viral glycoproteins (4, 5) and surface-bound immunoglobulin (6); a cytoplasmic, C-terminal peptide has been implicated in rapid transport of vesicular stomatitis virus G protein from the ER (7); and oligosaccharide phosphorylation triggers the sorting of lysosomal enzymes in mammalian fibroblasts (8–10).

A major question in the mechanism of secretion concerns the role of receptors in mediation of intracellular transport. The evidence for such receptors is entirely circumstantial. Two examples that are consistent with a receptor are that the rate of transport from the ER to the Golgi body varies for different proteins in the same cell (11–13), and that movement of retinol-binding protein from the ER requires retinol, presumably to alter the conformation of the binding protein (14). These cases could equally well be explained by selective negative influences on transport of slowly moving proteins. The challenge in this area is to develop evidence of a more positive nature, such as the identification of a signal or structure required for transport.

One method of defining localization signals in organellar proteins is the isolation of mutations in which these signals are defective so that the altered protein accumulates at some point along the normal transport pathway or is misdirected to some other cellular organelle. The most revealing application of this approach has been in the definition of important

[1] *Abbreviations used in this paper:* E and I fractions, external and intracellular fractions; endo H, endoglycosidase H; ER, endoplasmic reticulum; YPD medium, 1% Bacto-Yeast extract, 2% Bacto-peptone, and 2% glucose (YP medium, same but without glucose).

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aspects of the signal peptide in secretion of bacterial proteins (15–17). In eucaryotic cells, numerous examples of transport-deficient mutant forms of membrane and secretory proteins have been presented: IgA λ light chain (18), low-density lipoprotein receptor (19), Vaccinia Virus hemagglutinin (20), α_1-antitrypsin (21), vesicular stomatitis virus G protein (22–23; for review see reference 24). Defective proteins most often accumulate in the ER. Although some of these mutations may specifically affect a transport signal, it has been difficult to exclude the more trivial possibility that defective proteins are denatured or aggregated.

Retention of full enzyme activity in a secretion-defective mutant protein is a criterion that may be used to distinguish a transport lesion from more general perturbations of protein structure. With this in mind, we have examined invertase, an enzyme that follows the normal secretory pathway in yeast (25). The invertase gene (*SUC2*) has been cloned and sequenced (26, 27). Transformation of yeast cells with heavily mutagenized plasmid DNA has allowed a large number of *SUC2* mutants to be screened for accumulation of active invertase. In this report we describe the isolation, characterization, and precise mapping of two transport-specific mutations.

MATERIALS AND METHODS

Strains, Growth Conditions, and Materials: *Saccharomyces cerevisiae* strains SEY2101, SEY2102, and SEY5188 (all *suc2*-Δ9), and SEY5186 (*SUC2*, *sec18*), were described previously (28, 29). ISY1-22A (*ura3*-52, *leu2*-3, *leu2*-112, *his4*-519, *suc2*-Δ9, *pho*80-2) was constructed by standard genetic techniques.

pRB58, a YEp24-derived 2 μ plasmid (30) containing the *SUC2* gene, was obtained from M. Carlson (Columbia University) and has been described elsewhere (26). Plasmids pSEY125, pSEY124, and pSEY122, which carry *SUC2-lacZ* fusions, have also been described elsewhere (29). pSEY8 was derived from pUC8 (31) and also contains 2 μ DNA and the *URA3* gene (30).

YPD medium contained 1% Bacto-Yeast extract (Difco Laboratories Inc., Detroit, MI), 2% Bacto-peptone (Difco Laboratories Inc.), and 2% glucose. Wickerham's minimal medium (32) was used with various amounts of glucose or raffinose as a carbon source. For low sulfate medium, chloride salts replaced sulfate salts, and ammonium sulfate was added to the desired concentration. The absorbance of dilute cell suspensions was measured in a 1-cm cuvette at 600 nm in a Zeiss PMQII spectrophotometer; 1 OD_{600} unit of cells corresponds to 0.15 mg dry wt. Liquid cultures were grown in flasks or tubes with agitation, and experiments were initiated with exponentially growing cells at an OD_{600} of 0.5–4.

Other reagents were obtained as indicated: glucose oxidase, peroxidase, *o*-dianisidine, cycloheximide, cytochrome *c*, NADPH, flavin mononucleotide, triphenyltetrazolium chloride, tunicamycin, ATP, saponin, hydroxylamine, ergosterol, and tergitol Nonidet P-40 were from Sigma Chemical Co. (St. Louis, MO); $H_2{}^{35}SO_4$ and [^{3}H]leucine were from Amersham Corp. (Arlington Heights, IL); ENHANCE was from New England Nuclear (Boston, MA); Beta Max was from West Chem (San Diego, CA); IgG Sorb was from The Enzyme Center Inc. (Boston, MA); restriction enzymes and T4 DNA ligase were from Bethesda Research Laboratories (Gaithersburg, MD); oleate was from Merck and Co. (Rahway, NJ); BBL-Gas Pak anaerobic system catalyst and $CO_2 + H_2$ generator packs were from Fisher Scientific Co. (Pittsburgh, PA). Lyticase (fraction II) was used for production of spheroplasts. Endoglycosidase H (endo H) was obtained from P. Robbins (Massachusetts Institute of Technology). Acid phosphatase antibody was obtained from Simone Straumann-Böhni and Gottfried Schatz (Biozentrum, University of Basel).

Cytoplasmic invertase was purified by the method of Goldstein and Lampen (33) and used to raise antibody by the multiple intradermal injection method (34). Immune serum, which had a titer of about 1:16 to 1:32 against cytoplasmic invertase, was stored at −20°C (in 10 mM NaN_3) and used without further purification. Immune precipitation samples were mixed with aliquots of an extract prepared from strain SEY2102. This strain, which had no invertase cross-reacting material, was grown to late logarithmic phase in YPD medium; cells were centrifuged and resuspended in PBS (12.5 mM sodium phosphate, pH 7.0, 200 mM sodium chloride) at ~800 OD_{600} units of cells/ml and lysed by agitation for 5 min with glass beads (0.3–0.5 mm) in a Bead-Beater (Biospec Products, Bartlesville, OK). The extract was then adjusted to 1% SDS and heated in a boiling water bath for 6 min. A soluble fraction (30–40 mg protein/ml) was obtained by centrifugtion for 20 min at 12,000 *g*.

Mutagenesis and Mutant Screening: pRB58 plasmid DNA, purified by CsCl density sedimentation, was diluted into a solution of 0.4 M hydroxylamine, 0.05 M potassium phosphate, pH 6.0, at a DNA concentration of 125 μg/ml. After 60 min at 75°C, samples were chilled, and the DNA was precipitated with ethanol, resuspended in 10 mM Tris·HCl, pH 7.5, 2 mM EDTA, and precipitated with ethanol an additional two times. Yeast strain SEY2102 was transformed with mutagenized DNA by the lithium acetate procedure (35). Each petri plate (containing minimal medium without uracil) was spread with cells exposed to 2 μg of DNA.

Individual transformants were spotted onto selective plates (minimal medium without uracil) and screened for invertase secretion by one or both of two methods. The first screen involved a qualitative assay of invertase on a filter paper disk. Plates of 100 transformants were stamped in duplicate onto plates containing YP medium (no glucose) and incubated at room temperature for 3–4 h during which time invertase synthesis was derepressed. One of each pair of plates was inverted over a chloroform-soaked paper towel for 10 min. Whatman No. 1 filter disks (Whatman Laboratory Products Inc., Clifton, NJ) were bathed in a 2-ml solution that contained the invertase assay reagents: 0.1 M sodium acetate, pH 5.1, 0.125 M sucrose, 0.4 mM *N*-ethylmaleimide, 10 μg/ml peroxidase, 0.6 mg/ml *o*-dianisidine, and 0.05 mg/ml glucose oxidase. Chloroform-treated and untreated copies of the transformants were stamped onto damp filters. Within a few minutes at room temperature, a pink color developed over most of the patches. This procedure was used to identify mutant *suc2*-s1, which showed more intense staining in the chloroform-treated replica, but did not give sufficient resolution to detect the other mutants.

The other screening procedure involved analysis of intracellular invertase activity by electrophoresis on nondenaturing polyacrylamide gels. Cultures (5 ml) of individual transformants were grown to mid-logarithmic phase in minimal medium + 5% glucose. A mixture of 1 OD_{600} unit of cells from each of five cultures were centrifuged, washed with water, and incubated in minimal medium and 0.1% glucose for 3 h at 25°C. Cells were then washed with 10 mM sodium azide, and 1 OD_{600} was resuspended in 0.1 ml of 1.4 M sorbitol, 50 mM potassium phosphate, pH 7.5, 10 mM sodium azide, 80 mM 2-mercaptoethanol, and 50 U of lyticase. After 60 min at 30°C, spheroplasts that sedimented at 3,000 *g* for 10 min were lysed by resuspension in 50 mM potassium phosphate, pH 7.5, 10 mM sodium azide, 0.1% Triton X-100. An aliquot corresponding to 0.15 OD_{600} unit of cells was electrophoresed on a 5% polyacrylamide slab gel using a system described by Meyer and Matile (36) except without SDS. Invertase was localized in the gel, after incubation at 25°C for 20–60 min in 0.1 M sodium acetate (pH 5.0), 0.1 M sucrose, by the method of Gabriel and Wang (37). Pools containing mixtures of wild-type and *suc*$^-$ transformants showed primarily the rapidly and discretely migrating cytoplasmic invertase with faint staining in the region that contained glycosylated forms of invertase. In pools that showed more intense staining in this latter region, individual members were screened directly for invertase secretion and accumulation by the assay of Goldstein and Lampen (33) with minor modifications (38). A unit of invertase will release 1 μmol of glucose from sucrose per minute at 25°C.

Labeling, Immunoprecipitation, and Endo H Treatment: Cells were grown to mid-logarithmic phase in minimal medium + 5% glucose and 0.1 mM ammonium sulfate. 1 OD_{600} unit of cells was centrifuged, washed with water, and resuspended in fresh minimal medium + 0.1% glucose and no sulfate at 1 to 2 OD_{600} units of cells/ml. These conditions allowed derepression of invertase synthesis. After 20 min at 25°C, 250 μCi of carrier-free $H_2{}^{35}SO_4$ was added and cultures were incubated an additional 30 min. Labeled cells were washed and converted to spheroplasts as described in the previous section. Spheroplasts were centrifuged, the supernatant fractions were adjusted to 0.5% SDS, and the pellets were resuspended in 50 μl of 1% SDS. Samples were heated in boiling water for 3 min, diluted to 1 ml with PBS and 2% Triton X-100, and treated with 25–50 μl of IgG Sorb, prepared as described by the manufacturer, for 30 min at 0°C. IgG Sorb was removed by centrifugation at 12,000 *g* for 20 min and supernatant fractions were transferred to new microfuge tubes. Antibody was added (invertase serum, 4 μl/OD_{600} unit cell equivalent; acid phosphatase serum, 4 μl/OD_{600} until cell equivalent for spheroplasts, and 100 μl/OD_{600} unit cell equivalent for spheroplast supernatant fractions) and samples were incubated at 0°C for 2 h. Invertase immunoprecipitation samples were supplemented with 125 μl (4–5 mg protein) of nonradioactive extract from strain SEY2102; this served to compete for precipitation of unrelated proteins. IgG Sorb was added at 10–20 μl per 1 μl of serum, and immune complexes formed during 30 min at 0°C were sedimented at 12,000 *g* for 1 min. Complexes were washed twice with 0.5 ml of 2 M urea, 0.2 M sodium chloride, 0.1 M Tris·HCl, pH 7.5, 1% Triton X-100, and then twice with 0.5 ml of 1% 2-mercaptoethanol. Final pellets were resuspended in 25–50 μl of sample buffer (50 mM Tris·HCl, pH 6.8, 10% glycerol, 1% SDS, 2% 2-

mercaptoethanol, 0.01% bromphenol blue) and heated in boiling water for 3 min. After centrifugation in a microfuge for 5 min, the supernatant fractions were subjected to gel electrophoresis on 6% SDS polyacrylamide slab gels according to the procedure of Laemmli (39). Gels were fixed, in some cases prepared for fluorography with ENHANCE, dried, and allowed to expose Kodak X-Omat AR film at −70°C. Autoradiograms were quantified by scanning with an E-C Apparatus Corp. (St. Petersburg, FL) EC910 densitometer tracer.

Modifications of the standard procedure were used in certain experiments. In the kinetic analysis, cells were incubated with $H_2{}^{35}SO_4$ at 1 OD_{600} unit/ml for 5 min (or 10 min for labeling of acid phosphatase). Cultures were then adjusted to 0.5% glucose, 1 mM ammonium sulfate, 100 μg/ml cycloheximide, to initiate the chase period. Aliquots of 1 OD_{600} unit of cells were taken at various times and added to chilled tubes that contained 10 μl of 1 M sodium azide. Cells were washed once with cold 10 mM sodium azide and held at 0°C until the conclusion of the experiment. In the experiment in which acid phosphatase and invertase were both evaluated, samples were split and one-half of each was treated with the respective antiserum.

For treatment with endo H, aliquots of immunoprecipitated invertase, after solubilization in sample buffer and separation from IgG Sorb, were diluted twofold and adjusted to 0.1 mg/ml BSA, 0.1 M sodium acetate, pH 5.1. Samples were incubated with 0.5 mU of endo H overnight at 37°C. After being heated in boiling water, samples were subjected to SDS gel electrophoresis.

N-Terminal Analysis of Mutant and Wild-type Invertase: For N-terminal analysis of secreted invertase, strain SEY2102 containing pRB58 with either *SUC2* or the s1 mutant was derepressed for invertase synthesis (4 OD_{600} units of cells) in 2 ml of minimal medium with no sulfate and 0.1% glucose. After 10 min at 25°C, 2 mCi of $H_2{}^{35}SO_4$ were added to each sample for 60 min at 25°C, and a chase period was initiated as described earlier for the kinetic analysis. Cells were centrifuged after a 60-min chase period, washed with 10 mM sodium azide, and converted to spheroplasts. Invertase in the E fraction was immunoprecipitated as described earlier, using anti-invertase antibody that had been affinity-purified by adsorption to and elution from cytoplasmic invertase-conjugated to Sepharose 4B. The precipitates were solubilized in 30 μl of sample buffer and separated from IgG Sorb by centrifugation; 1 μl of each was subjected to SDS gel electrophoresis to confirm the purity of the labeled samples. The remaining samples were extracted twice with 1 ml acetone/triethylamine/acetic acid (90:5:5) and then three times with acetone. Each extraction was incubated for 20 min at 0°C and then centrifuged at 12,000 *g* for 10 min. The final pellets were resuspended in 50 μl of 10 mM ammonium bicarbonate, 0.02% SDS. Samples were subjected to sequential Edman degradation using an Applied Biosystems (Foster City, CA) 470A gas-phase protein sequencer. Material released in each of the first 15 cycles was measured for ^{35}S counts per minute.

For N-terminal analysis of ER-localized invertase, 5 OD_{600} units of SEY5188 (*sec18*) containing pRB58 with either wild-type or s1 mutant *SUC2* genes were transferred to 1 ml of minimal medium with 0.1% glucose, no sulfate, and 4 μM leucine. After 10 min at 25°C, 250 μCi of [^{3}H]leucine and 2 mCi $H_2{}^{35}SO_4$ were added to each sample. Cells were incubated for 60 min at 37°C, washed with 10 mM sodium azide, and converted to spheroplasts. Spheroplast pellet fractions were lysed, and invertase was immunoprecipitated with affinity-purified antibody and treated with endo H as described earlier. Samples were subjected to SDS gel electrophoresis on a 6% polyacrylamide gel that had been polymerized overnight and prerun for 5 min. The gel was dried without fixation and subjected to autoradiography. Protein bands of interest were excised from the gel and electroeluted into dialysis tubing overnight at 4°C in 0.1 mM Tris-HCl, pH 8.0, 0.1% SDS, 0.1 mM sodium thioglycolate. Electroeluted samples were lyophilized to dryness and extracted twice with acetone/triethylamine/acetic acid/water (88:5:5:2) and three times with acetone as described earlier. The final pellets were resuspended in 10 mM ammonium bicarbonate, 0.02% SDS. Samples were subjected to sequential Edman degradation, and 15 cycles were counted for ^{35}S and ^3H. In the samples of wild-type invertase, ~35,000 cpm of ^{35}S was released at the indicated cycle and ~2,000 cpm of ^3H was released. s1 mutant invertase samples released ~16,000 cpm of ^{35}S at the indicated cycle, and 1,000–1,500 cpm of ^3H was released at each of the indicated cycles.

Release of Invertase by Saponin: To test the solubility of the accumulated invertases, 50 OD_{600} units of SEY2102 cells, containing an integrated copy of either *SUC2*-s1 or *SUC2*-s2, and SEY5186 (*sec18 SUC2*) were collected by centrifugation and washed once with water. Cells were resuspended in 50 ml of minimal medium and 0.1% glucose. After 2 h at 37°C, 50 OD_{600} units were collected by centrifugation, washed with 10 mM sodium azide, and converted to spheroplasts as described earlier, except at 50 OD_{600} U/ml and with 5,000 U/ml of lyticase. After 60 min incubation at 30°C, spheroplasts were collected by centrifugation for 5 min at 3,000 *g* and washed once with spheroplast buffer without lyticase and 2-mercaptoethanol. Spheroplasts were then lysed in 50 mM KH_2PO_4, pH 7.5, 10 mM NaN_3, a treatment that retained ~80% of wild-type invertase in *sec18* in a latent, sedimentable form. Aliquots of the lysed spheroplasts were adjusted to a range of saponin concentrations, incubated at 4°C for 60 min, and then centrifuged at 133,000 *g* for 12 min in a Beckman airfuge (Beckman Instruments, Inc., Palo Alto, CA) with an A100 fixed-angle rotor. Supernatant fractions were removed and saved. Pellet fractions were resuspended to the original volume in 50 mM KH_2PO_4, pH 7.5, 10 mM NaN_3, 0.1% Triton X-100. Fractions were assayed for protein concentration, invertase activity, and cytochrome *c* reductase activity according to published procedures (protein [40], invertase [33], cytochrome *c* reductase [41]).

Recombinant DNA and Marker Rescue Mapping: Plasmid purification, agarose gel electrophoresis, transformation of bacteria, and other DNA manipulations were performed by standard methods (42).

Wild-type, s1, and s2 *SUC2* genes were subcloned into YIp5 (30) by ligation of a 4.5 base pair *Xho*I to *Cla*I fragment of the *SUC2* insert into YIp5 cut with *Sal*I and *Cla*I. Uncut YIp5 containing wild-type and mutant *SUC2* genes were then transformed into SEY2102. *URA*$^+$ transformants resulting from integration of YIp5 into the genome were picked and assayed for invertase secretion.

For construction of mutant-wild type hybrid genes, *SUC2* was first subcloned by inserting the *Hin*dIII fragment of pRB58 into the unique *Hin*dIII site of pSEY8 (see Fig. 6). The 800 base *Eco*R1-*Bam*H1 fragment in this pSEY8 construct was then replaced with the 1,600 base *Eco*R1-*Bam*H1 fragment of *SUC2*-s1, -s2, or -s3 from mutant pRB58 plasmids. Hybrids then contained the 5′ half of a mutant gene and the 3′ half of wild-type *SUC2*.

For marker rescue mapping, *SUC2*-s1, -s2, and -s3 *Hin*dIII fragments were subcloned into the pSEY8 *Hin*dIII cloning site, and the resulting plasmids were introduced into yeast strain SEY2102 (*α suc2-Δ9*). SEY2102 (*a suc2-Δ9*) was transformed with the *SUC2-lacZ* fusion vectors pSEY125, pSEY124, and pSEY122 (29). Strains were mated pairwise on YPD plates, and after 1–2 d the mating patches were resuspended in water and spread on minimal medium plates +2% sucrose, 0.02 mg/ml ergosterol, 0.25 mg/ml oleate, 2.5 mg/ml Tergitol Nonidet P-40, and 20 μg/ml uracil. Plates were incubated at 30°C in an anaerobic jar using the BBL-Gas Pak anaerobic system. These conditions allowed growth only of *SUC*$^+$ recombinants that were picked after 5–6 d. *SUC*$^+$ colonies were streaked on fresh plates and grown selectively for an additional 4 d. Single colonies representing a number of original recombinants were grown in liquid medium, and secretion and accumulation of invertase were assessed as before.

DNA Sequencing: Preparations of each of the mutant *SUC2* plasmids were digested with *Bam*H1 and *Hin*dIII. The 776 base fragment that contained the 5′ half of each mutant *SUC2* gene was purified by gel electrophoresis in and extraction from low melting temperature agarose (43). M13 mp8 or M13 mp9 RF (replicative form) DNA was digested with *Hin*dIII and *Bam*H1, and 30 ng of this DNA was reacted with 200 ng of the *SUC2* purified fragment in the presence of T4 DNA ligase. Ligated DNA was used to transfect *E. coli* JM103 (44). Single-stranded DNA was isolated from cultures grown from individual recombinant phage plaques and used as template for DNA sequencing by the dideoxy chain termination technique (45). M13 mp8 and M13 mp9 have *Hin*dIII and *Bam*H1 cloning sites in opposite orientations. M13 mp8 provided coding strand sequence for about 450 bases from the *Hin*dIII site in the signal sequence coding region, while M13 mp9 provided 450 base pairs of sequence on the noncoding strand from the *Bam*H1 site in the middle of the *SUC2* gene.

RESULTS

Mutagenesis of Cloned SUC2 Gene

A 2 μ yeast plasmid containing the *SUC2* gene and a selectable marker gene (*URA3*) was mutagenized in vitro with hydroxylamine which produces C to T transitions. Mutant DNA was introduced by transformation into a *ura3* strain that contained a deletion in the *SUC2* gene (28). Hydroxylamine treatment was adjusted so that 10–15% of the plasmids that were recovered by transformation contained *SUC2* mutations resulting in no active invertase. The efficiency of transformation by DNA mutagenized at this level was reduced twofold for yeast and 10^4-fold for *E. coli*.

In a sampling of 60 independent *suc*$^-$ mutants, 31 failed to make any invertase immunoreactive protein, which, in most cases, was due to major rearrangements in the plasmid DNA. Of the remaining 29 mutants in which immunoreactive ma-

terial was detected, six produced forms with electrophoretic mobilities characteristic of invertase accumulated in the endoplasmic reticulum (46). These mutations may produce denatured forms of invertase that can not be transported from the ER.

To identify mutations that affect transport but not correct folding of the invertase polypeptide, we screened 6,000 independent transformants by procedures that detect intracellular accumulation of active invertase. Four were found that fit this criterion, and the extent of invertase accumulation is shown in Table I. Cells transformed with the normal or mutant (*SUC2*-s1 to -s4) plasmids were derepressed for invertase synthesis, converted to spheroplasts, and the released (periplasmic) and sedimentable (intracellular) activity was measured. With the exception of mutant s4, nearly normal levels of invertase were produced (Table I, lines 1–5). All of the mutants showed at least fivefold accumulation of intracellular invertase, with s1 the most dramatic at 30-fold. Invertase derepressed under steady-state conditions of growth, in raffinose, showed the same accumulation behavior (Table I, lines 6–8). Despite the reduced rate of invertase transport, cells carrying the mutant plasmids grew normally on sucrose-selective medium.

Cytoplasmic invertase, a nonsecreted protein product of an alternative *SUC2* transcript (26), was examined by electrophoresis of spheroplast extracts on a nondenaturing gel system. Mutants s1, s2, and s3 produced normal amounts of this enzyme (not shown). Mutant s4 was eliminated from further consideration because the low amount of periplasmic invertase produced was less stable and cytoplasmic invertase was inactive.

Cells transformed with the multicopy *SUC2* plasmid produced about 10-fold more invertase than was detected in normal cells with a single-chromosomal *SUC2* gene. To test whether overproduction of the mutant invertase contributed to the secretion-defective phenotype, *SUC2* mutant genes were subcloned into the integrating plasmid YIp5 and recombined into the yeast genome. Whereas the amount of invertase dropped 10-fold, the extent of accumulation was unaffected (Table I, lines 9–11). These results also showed that the mutations were linked to *SUC2*.

Mutants Defective in Transport of Core-glycosylated Invertase

Invertase transport intermediates have been identified in pleiotropic *sec* mutants that are blocked at successive stages in the secretory pathway. Mutants such as *sec18*, in which transport from the ER to the Golgi body is blocked, represent the earliest stage where an active secretory form of invertase is detected. SDS gel electrophoresis of invertase accumulated in *sec18* reveals several discrete species of around 80 kd, corresponding to core-glycosylated molecules. Subsequent oligosaccharide modifications in the Golgi body produces heterogeneous, low electrophoretic mobility forms of invertase (46).

To identify the site of accumulation of mutant invertase, we labeled cells (wild type and *sec18*) transformed with plasmids carrying wild-type, *SUC2*-s1, -s2, or -s3 mutant genes for 30 min with $^{35}SO_4^{2-}$, converted them to spheroplasts, and evaluated the periplasmic (external, E) and intracellular (I) fractions by immune precipitation and SDS gel electropho-

TABLE I

Accumulation of Active Invertase in SUC2 Mutants

Line	Plasmid/*SUC2* type	Derepressed intracellular invertase*	Periplasmic invertase	Total activity	Fold accumulation‡
		Units/OD cells		*% wild type*	
1	pRB58/*SUC2*	0.40	1.71	100	1
2	pRB58/*SUC2*-s1	1.96	0.28	106	30
3	pRB58/*SUC2*-s2	1.12	0.76	89	6.7
4	pRB58/*SUC2*-s3	1.13	0.87	95	5.7
5	pRB58/*SUC2*-s4	0.52	0.43	45	5.2
6	pRB58/*SUC2*	0.04	0.77	100	1
7	pRB58/*SUC2*-s1	0.60	0.35	117	34
8	pRB58/*SUC2*-s2	0.16	0.53	85	6
9	YIp5/*SUC2*	0.03	0.29	100	1
10	YIp5/*SUC2*-s1	0.15	0.05	63	33
11	YIp5/SUC2-s2	0.27	0.22	150	12
12§	pRB58/*SUC*-s1 / *Eco-Bam* hybrid	0.74	0.12	62	36
13	pRB58/*SUC2*-s2 / *Eco-Bam* hybrid	0.71	0.90	116	6
14	pRB58/*SUC2-s3* / *Eco-Bam* hybrid	0.55	0.83	99	5.1

* SEY2102 was transformed with the indicated plasmids. Except in the experiment in lines 6–8, cells were grown to mid-log phase in selective minimal medium and 5% glucose, washed, and derepressed for 3–4 h at 25°C in minimal medium and 0.1% glucose. Cells were converted to spheroplasts and centrifuged, and the supernatant (periplasmic) and pellet (intracellular) fractions were assayed for invertase activity. A constant background of cytoplasmic invertase activity was subtracted to give the derepressed intracellular level. Cells in the experiment in lines 6–8 were grown in minimal medium and 2% raffinose.

‡ The ratio of derepressed intracellular to periplasmic activity for mutant *SUC2* alleles divided by the ratio for wild-type *SUC2*.

§ Results of a separate experiment with values normalized to assays of pRB58/*SUC2* done in parallel.

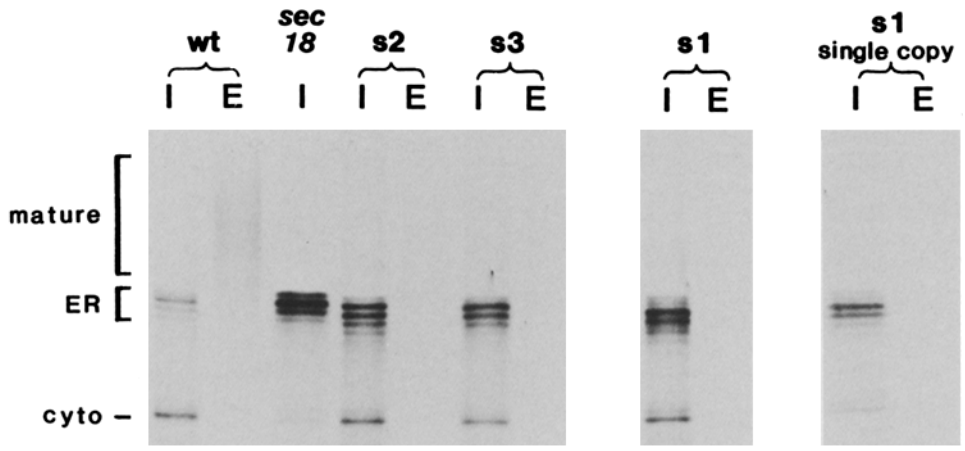
resis. After this labeling period in wild-type cells containing pRB58, the cytoplasmic invertase and some of the ER transit form were found in the I fraction, while much of the invertase was highly glycosylated and in the E fraction (Fig. 1). The ER transit forms accumulated in *sec18* were similar to those found in wild-type cells. *SUC2*-s1, -s2, and -s3 produced nearly exclusively ER transit forms of invertase. Mutant s1 (and s2, not shown) showed the same behavior when integrated into the chromosome (single copy). Unlike the pleiotropic *sec* mutants, the *SUC2* transport mutants were blocked to the same extent at 25° and 37°C (not shown). Hence, by comparison with the behavior of *sec18*, the *SUC2* mutants appear to be nonconditionally defective in transport from the ER, or possibly from a compartment between the ER and the site of outer chain carbohydrate addition.

In experiments with longer radiolabeling periods, highly glycosylated, secreted invertase was detected in cells that contained the s1, s2, and s3 mutant alleles. The possibility that these mutants were not absolutely defective in transport allowed additional definition of the stage in the pathway and of the specificity of the defects.

If the transport-defective invertase is delayed in the secretory pathway before or concurrent with the *SEC18* dependent step, then invertase that accumulates at 25°C will not progress beyond the *sec18* block at 37°C. On the other hand, if mutant invertase is delayed beyond the *SEC18* step, progress will not be hindered in *sec18* cells at 37°C. *sec18* cells, transformed with a plasmid carrying *SUC2*-s3, were labeled with $^{35}SO_4^{2-}$ for 10 min at 24°C (*sec* permissive temperature). Culture aliquots were subsequently incubated for 30 and 60 min at 24° and 37°C (*sec* nonpermissive temperature) in the presence of cycloheximide. Intracellular and external invertase was examined by immunoprecipitation (Fig. 2). After the pulse, only core-glycosylated invertase in the I fraction was observed. With increasing chase time at 24°C, heterogeneous glycosylated material was detected in the E fraction, with a corresponding decline in core-glycosylated protein in the I fraction. No further glycosylation or secretion was seen when the chase was conducted at 37°C. This pattern was qualitatively similar in mutants s1 and s2, suggesting that all the invertase defects delay transport before or at the *SEC18* step. Control experiments with wild-type cells containing mutant invertase showed secretion of about one-half of the s1 invertase, and all of the s2 mutant protein, during the 60-min chase at 37°C.

Differences among the invertase mutants were uncovered by a more detailed analysis of the kinetics of secretion at 24°C. Cells that carried plasmids with wild-type, s1, or s2 invertase were pulse-labeled for 5 min. At various times during subsequent incubation in the presence of cycloheximide, samples were withdrawn and the distribution of immunoreactive forms of invertase was evaluated. Fig. 3 shows the percentage of radiolabeled invertase (open symbols, core glycosylated; closed symbols, heterogeneous forms) that remained intracellular at the indicated times of chase. In the pulse label, 60% of the wild-type invertase was core glycosylated and intracellular, whereas the highly glycosylated material was equally divided between intracellular and secreted. Conversion of core- to highly-glycosylated invertase occurred with a $t_{1/2}$ of ~1.5 min for the wild-type, ~75 min for mutant s1, and ~10 min for mutant s2 (same as s3, not shown). A semilogarithmic plot of the data for the mutants revealed a single kinetic constant for conversion of invertase. In each case, the total amount of labeled invertase remained constant during the chase period. A transient increase in highly glycosylated intracellular invertase was seen early in the chase period of wild-type protein, while mutant s1 and s2 showed negligible intracellular levels of this form. These results suggest that mutants s1 and s2 are distinct, and that secretion is specifically delayed in the ER with no observable effect on subsequent transport from the Golgi body.

Additional evidence for the specificity of the invertase mutations came from an analysis of acid phosphatase secretion and growth rates of strains that contained the mutant *SUC2* plasmids. A strain that carries the *pho80*-2 mutation, and therefore constitutively makes and secretes acid phosphatase (47), was transformed with wild-type or mutant *SUC2* plasmids. Cells were derepressed for invertase expression and labeled for 10 min at 25°C, and samples were withdrawn at intervals during a chase in the presence of cycloheximide. The distribution of radiolabeled forms of acid phosphatase was



FIGURES 1 Immunoprecipitation and electrophoresis of wild-type and mutant invertases. SEY2102 cells transformed with pRB58 containing either wild-type or mutant *SUC2* genes and SEY5188 (*sec18*) containing pRB58 were derepressed for invertase expression and labeled with $^{35}SO_4^{2-}$ for 30 min. Samples were separated into spheroplast pellet (*I*) and spheroplast supernatant (*E*) fractions and immunoprecipitated with invertase antiserum. Immunoprecipitates were analyzed by SDS PAGE and autoradiography. *mature*, highly glycosylated invertase; *ER*, core-glycosylated invertase characteristic of endoplasmic reticulum forms; *cyto*, cytoplasmic invertase.

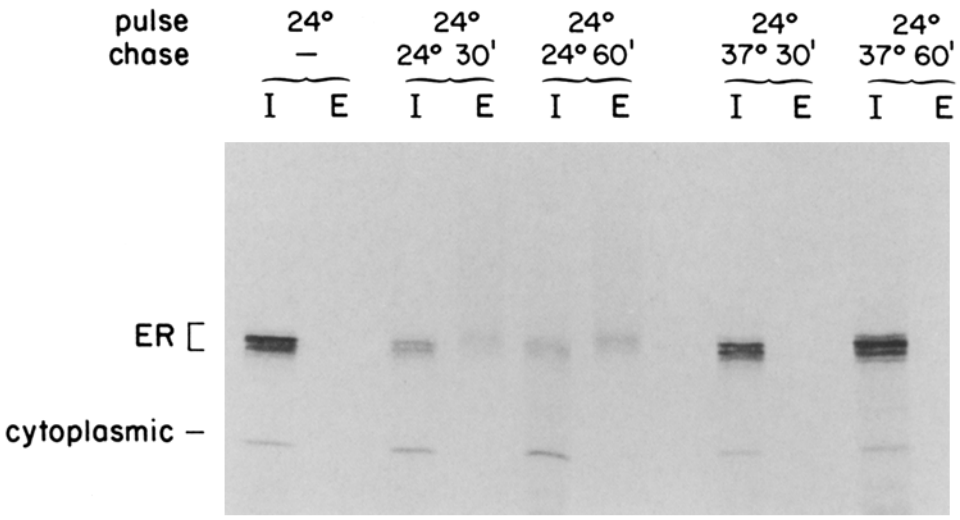


FIGURE 2 Secretion of s3 mutant invertase blocked in *sec18* at 37°C. SEY5188 (*sec18*) cells transformed with pRB58 that contained *SUC2*-s3 were derepressed for invertase expression and labeled with $^{35}SO_4^{2-}$ for 10 min at 25°C. Immediately after initiation of the chase period by addition of glucose, unlabeled sulfate, and cycloheximide, an aliquot of the culture was transferred to 37°C and the rest was kept at 25°C. Aliquots were taken at various times, separated into spheroplast pellet (*I*) and spheroplast supernatant (*E*) fractions, and immunoprecipitated with invertase antiserum. Immunoprecipitates were analyzed by SDS PAGE and autoradiography.

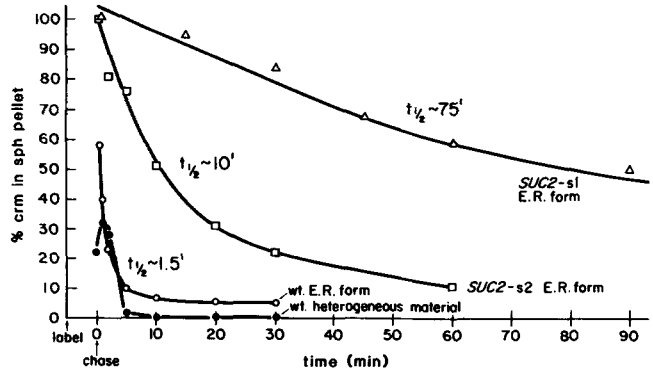


FIGURE 3 Kinetics of wild-type and mutant invertase secretion. SEY2102 cells that contained either the wild-type or s2 mutant *SUC2* genes were derepressed for invertase expression and labeled for 5 min with $^{35}SO_4^{2-}$. Chase was initiated with the addition of glucose, unlabeled sulfate, and cycloheximide. Aliquots were taken at various times, separated into spheroplast pellet and supernatant fractions, and immunoprecipitated with invertase antiserum. Immunoprecipitates were subjected to SDS PAGE and fluorography. Cross-reacting material was quantified by densitometric scanning.

evaluated by immunoprecipitation and SDS gel electrophoresis. As with invertase, acid phosphatase undergoes a core- to highly-glycosylated conversion after transport to the Golgi body (46). Core-glycosylated acid phosphatase was converted and secreted at the same rate in cells that were producing wild-type or mutant invertase (not shown). In the same samples, the pattern of invertase glycosylation and secretion was identical to that seen in Fig. 3. Furthermore, the invertase mutants did not interfere with any essential aspect of growth; doubling times in derepressing media were the same for cells carrying wild-type or *SUC2* mutant plasmids.

Physical Properties of the Mutant Invertases

Delayed secretion of mutant invertase could be accounted for by aberrant subunit interaction or aggregation within the lumen of the ER. This possibility was tested by native gel electrophoresis of invertase released from membranes by treatment with Triton X-100. Secreted invertase, detected with an activity stain, migrates as a heterogeneous mixture, whereas enzyme accumulated in the ER (such as in *sec18*) displays four discrete species which appear to be multimeric forms of the active dimer (46). For comparison, wild-type and *sec18* cells containing *SUC2*, *SUC2*-s1 (on a multicopy plasmid or integrated), -s2, or -s3 were derepressed for invertase synthesis and fractionated as before. Fig. 4 shows the pattern described above for wild-type and *sec18* cells. The intracellular enzyme produced by mutants s2 and s3 migrated as did invertase in *sec18*. Mutant s1, however, appeared aggregated with a heterogeneous and low electrophoretic mobility. A pattern more like that of *sec18* was seen with cells that contained a single copy of mutant s1, although most of the invertase was concentrated in the lowest mobility forms.

While Triton X-100 quantitatively released the ER-accumulated forms of invertase, at least one ER integral membrane protein, NADPH-cytochrome *c* reductase, was also solubilized. To address the issue of invertase solubility in the ER lumen, saponin, a detergent that permeabilizes but does not solubilize the ER membrane, was used to examine release of invertase activity from the membrane fraction. Spheroplasts made from cells (wild type or *sec18*) that had accumulated normal or mutant invertase were osmotically lysed under conditions that retained invertase in a sedimentable, presumably ER-bounded form. Extracts were mixed with various amounts of saponin at 0°C, and the amounts of sedimentable protein, NADPH-cytochrome *c* reductase, and invertase were assessed. Fig. 5 shows that very little protein (*top*) and no NADPH cytochrome *c* reductase (*middle*) were solubilized by saponin concentrations as high as 0.6 mg/mg protein. Wild-type invertase accumulated in *sec18* and s2 mutant invertase were readily released (70–80% solubilized; Fig. 5, *bottom*) by saponin at 0.2–0.3 mg/mg protein. In contrast, only ~35% of the s1 mutant invertase was released by saponin. S1 mutant enzyme that resisted saponin extraction was solubilized with 0.1% Triton X-100 and compared with the saponin-released invertase by native gel electrophoresis. Heterogeneous, low-mobility invertase was enriched in the saponin pellet fraction and discrete, higher-mobility forms were recovered in the saponin soluble fraction (not shown). Hence, by the native gel electrophoresis and saponin solubility criteria, s1 mutant invertase appears to be aggregated and to some extent associated with the ER membrane, whereas the

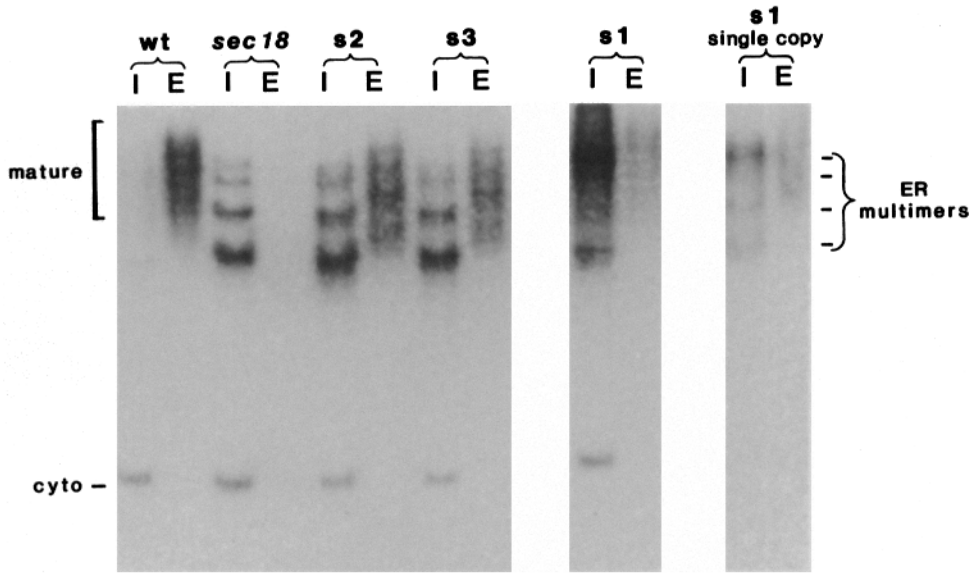


FIGURE 4 Nondenaturing gel electrophoresis of wild-type and mutant invertase. SEY2102 cells containing either wild-type or mutant *SUC2* genes and SEY5188 (*sec18*) cells containing the wild-type *SUC2* gene were derepressed for invertase expression for 3 h and separated into spheroplast pellet (*I*) and spheroplast supernatant (*E*) fractions. Samples were subjected to nondenaturing gel electrophoresis, and the gel was stained for invertase activity.

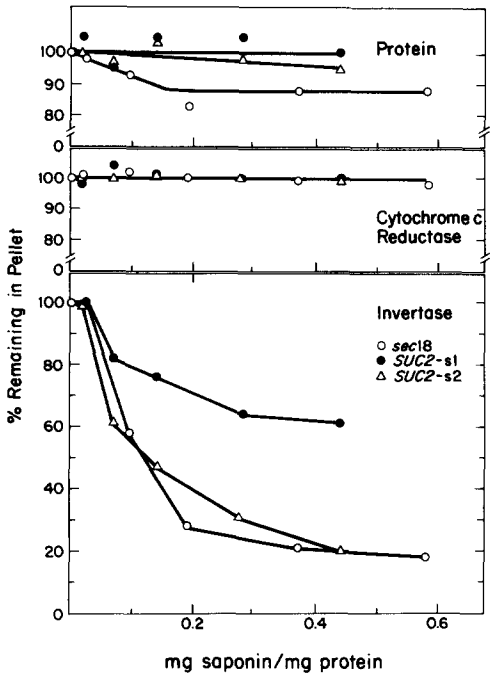


FIGURE 5 Saponin solubilization of accumulated invertase. SEY2102 cells that contained the mutant *SUC2* genes and SEY5188 (*sec18*) cells with the wild-type *SUC2* gene were derepressed for invertase expression and converted to spheroplasts. Sedimented spheroplasts were lysed by osmotic shock. Aliquots were treated with different saponin concentrations and centrigued at 133,000 *g*. Pellet and supernatant fractions were assayed for invertase activity (*bottom*), NADPH-cytochrome c reductase activity (*middle*), and total protein (*top*). Values represent the fraction sedimentable relative to the no detergent control. ○, *sec18*; ●, *SUC2*-s1; △, *SUC2*-s2.

s2 mutant enzyme is indistinguishable from normal invertase accumulated in the ER lumen. s2 and s3 mutant enzymes were identical in these characteristics. In other experiments, the mutant invertases had heat stability properties similar to those of the wild-type enzyme.

Mapping and Sequencing the SUC2 Mutations

Since the *SUC2* mutants were derived from DNA that was heavily mutagenized, it was likely that the isolates contained multiple mutations. For this reason, it was essential to localize the phenotypically important mutations by genetic mapping.

We localized the transport-deficient mutations to the 5′ half of *SUC2* by subcloning a 1,600 base pair *Eco*R1-*Bam*H1 fragment from the mutant DNA, containing about 770 bases of the 5′ coding region, into a vector that contained the 3′ half of wild-type *SUC2*. Donor and recipient plasmids are diagrammed in Fig. 6. Yeast transformants that contained the hybrid constructs showed the same invertase accumulation phenotype of the parents (Table I, lines 12–14). The accumulated material was examined by nondenaturing and SDS gel electrophoresis, and with one minor exception, the original phenotypes were reproduced. In the original s1 isolate, cytoplasmic invertase migrated more slowly than normal on a nondenaturing gel (Fig. 4). The *Eco*R1-*Bam*H1 hybrid of mutant s1 produced a normal cytoplasmic invertase (not shown), suggesting that the 3′ half of the original isolate contained at least one additional mutation that had no detectable influence on invertase transport.

To localize the transport mutations more precisely within the 5′ coding region, we devised a form of marker rescue recombinant analysis. *Hin*dIII fragments from plasmids carrying *SUC2*-s1, -s2, and -s3 were subcloned into the *Hin*dIII site of pSEY8 (Fig. 6). These constructs were missing the 5′ regulatory sequences and the first 10 bases coding for the secreted form of invertase. Cells transformed with these plasmids were mated with cells carrying another plasmid that contained one of a series of gene fusions in which a portion of the wild-type *SUC2* 5′ regulatory and coding sequence was fused to a nearly full-length fragment of the *E. coli lacZ* gene (29). Neither construct allowed invertase synthesis; consequently, anaerobic growth of diploid strains on sucrose provided a selection for recombinants that generated functional invertase. Since the presence of even the s1 mutant invertase allowed growth on sucrose, wild-type and transport-deficient recombinants were generated. Wild-type recombinants could be generated only if the region containing the transport mu-

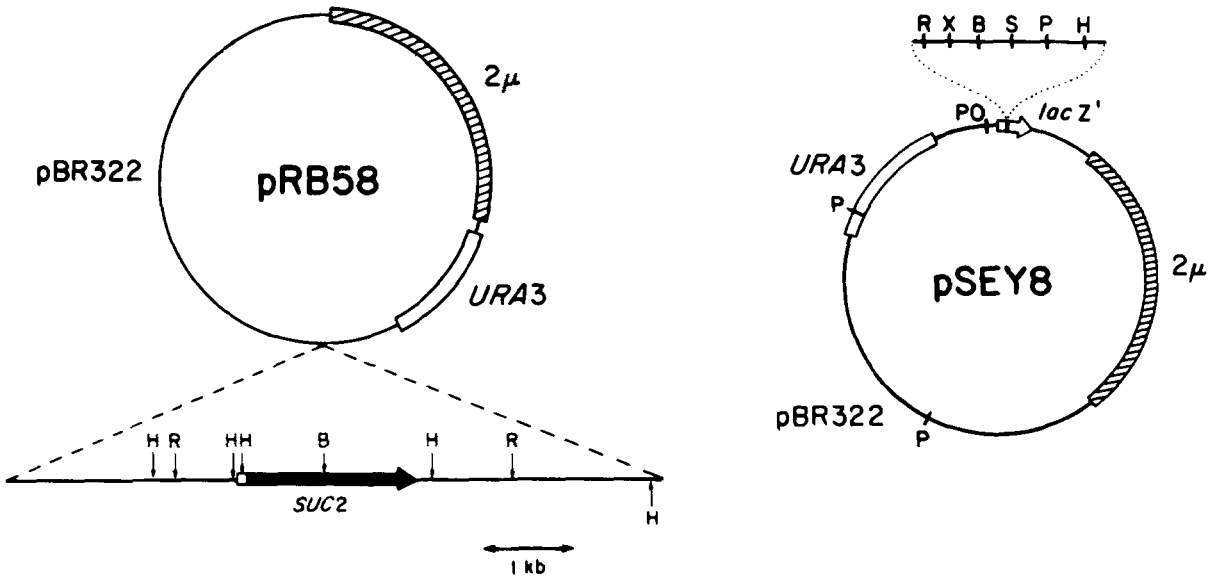


FIGURE 6 Plasmids for hybrid *SUC2* mapping. pRB58 contained the *SUC2* gene and pSEY8 provided a region with many convenient restriction sites. *H*, *Hin*dIII; *R*, *Eco*R1; *B*, *Bam*H1; *X*, *Sma*1; *S*, *Sal*1; *P*, *Pst*1.

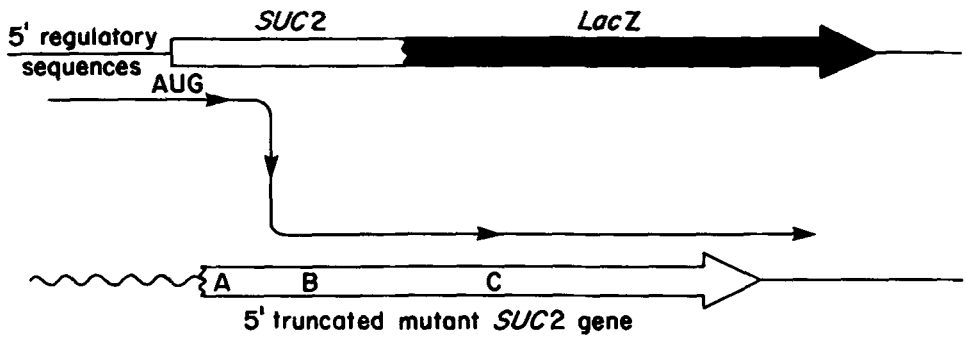


FIGURE 7 Marker rescue recombinational mapping of *SUC2* mutants. The strategy for recombinational mapping of the *SUC2* mutations is outlined. Plasmids carried either a *SUC2-lacZ* fusion (*top*) or a 5′-truncated mutant *SUC2* gene (*bottom*). *A*, *B*, and *C* are hypothetical mutations and the long arrow between the two plasmids is one possible *SUC2*$^+$ recombinant.

tation was represented on the *SUC2 lacZ* fusion. The ratio of wild-type and mutant recombinants was an approximate measure of the position of the mutation within the region of homology. This point is illustrated in Fig. 7: Mutations in region *A* would give mainly wild-type recombinants, in *B* would yield a mixture of mutant and wild-type, and in *C* would produce only mutant recombinants. Fig. 8 presents data on the percentage of wild-type recombinants generated in crosses between each mutant and hybrid genes that contained 150, 600, or 800 bases of *SUC2*. Mutant s1 showed a high percentage of wild-type recombinants even with the smallest *SUC2* fusion tested. Mutants s2 and s3 showed wild-type recombinants only with the next larger *SUC2* fusion. The possibility that transport mutations are 5′ to the *Hin*dIII site in the coding sequence is eliminated by these data. Approximate map positions for these mutations are indicated in Fig. 8. Additional mutations required for the transport defect could reside between the marker rescue site and the *Bam*H1 site in the middle of the gene.

The transport mutations were defined precisely by DNA sequence analysis of the 776 base *Hin*dIII-*Bam*H1 fragment from each mutant plasmid. *SUC2*-s1 contained two mutations in this region. One silent mutation resulted in exchange of phe codons at amino acid position 150 (TTC to TTT). The important mutation produced a change in the final codon of the signal peptide from ala to val (GCA to GTA). Although *SUC2*-s2 and -s3 mutations were derived from separate samples of mutagenized DNA, they both contained the same single substitution resulting in ile in place of thr (ACT to ATT) at position +64 in mature invertase. Two segments of the invertase protein sequence in the area of mutations s1 and s2 are shown in Fig. 9. A close correspondence was found between the map position determined by marker rescue recombinant analysis and DNA sequencing of the mutations.

Signal Peptide Processing of Mutant Invertase

Secreted invertase is processed from a precursor that contains a 19 amino acid signal peptide (48). Since valine is not found on the N-terminal side of signal peptide cleavage sites (49), *SUC2*-s1 mutant invertase could be defective in cleavage by signal peptidase. Retention of the signal peptide on mutant invertase was assessed by treatment of accumulated and secreted material with endo H. *sec18* and wild-type cells containing the *SUC2*, -s1, or -s2 plasmids were radiolabeled with

	SUC2-s1	*SUC2*-s2	*SUC2*-s3
pSEY125 (150 bp)	80% wt (40)	0% wt (47)	0% wt (36)
pSEY124 (600 bp)	100% wt (12)	~90% wt (28)	~70% wt (30)
pSEY122 (800 bp)	100% wt (5)	100% wt (2)	N.D.

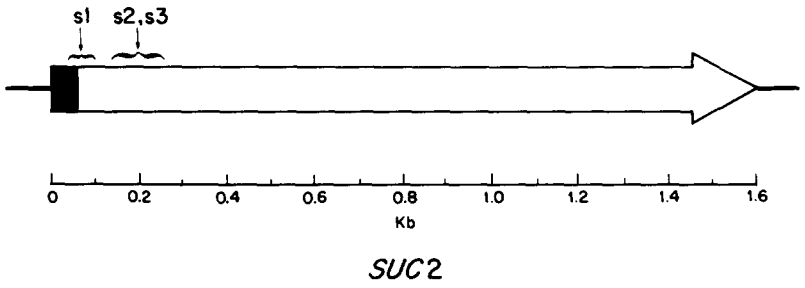


FIGURE 8 Mutations map to a region of ≤250 base pairs at the 5′ end of the *SUC2* gene. SEY2102 cells containing 5′ truncated constructs of the mutant *SUC2* genes were mated with SEY2101 containing three different size classes of *SUC2-lacZ* fusions. PSEY125 contained 150 bases, pSEY124 contained 600 bases, and pSEY122 contained 800 bases of *SUC2* coding sequence. The table gives the percentage of wild-type recombinants from each cross. The number in parenthesis is the number of recombinants analyzed for each cross. The sketch shows the approximate positions of the mutations predicted by the mapping data.

```
                                                                  signal
                                                                  cleavage
          -19                          -10                          ↓ 1
SUC2      Met-leu-leu-gln-ala-phe-leu-phe-leu-leu-ala-gly-phe-ala-ala-lys-ile-ser-ala-ser-met...

                                                                    ↓
SUC2-s1                                                            val


                          60                              70
SUC2   ···gly-his-ala-thr-ser-asp-asp-leu-thr-asn-trp-glu-asp-gln-pro-ile···

                                    ↓
SUC2-s2                             ile
```

FIGURE 9 Amino acid changes in *SUC2* transport mutants.

$^{35}SO_4^{2-}$ for 30 min and converted to spheroplasts, and the I and E fractions were treated with invertase antibody. Samples of the immunoprecipitates were treated with endo H under conditions where all the oligosaccharides are cleaved from invertase. Untreated and deglycosylated invertase samples were compared by SDS gel electrophoresis. Endo H treatment produced a form with an apparent M_r of 58,500 for normal invertase accumulated in *sec18*, and s2 invertase in normal cells (Fig. 10). The difference of ~2.5 kd between this species and the cytoplasmic invertase was expected from the 10–12 GlcNAc residues that remain after endo H treatment. Two forms of deglycosylated s1 invertase of approximately equal abundance were seen in the I fraction. One was the 58.5-kd species and the other was a 61-kd form corresponding to an increment expected for retention of the signal peptide. The same 2.5-kd size difference was seen in a comparison of unglycosylated s1 and wild-type invertase produced in tunicamycin-treated cells (not shown). By this criterion, signal peptide processing was normal for mutant s2 but defective for s1 mutant invertase.

After a chase period of 2 h, invertase secreted in mutant s1 was in the usual highly glycosylated form. Only the 58.5-kd species was detected when this material was treated with endo H (Fig. 10). Since the immunoreactive invertase was recovered in good yield after the chase period, some of the glycosylated precursor must have been proteolytically processed en route to the periplasm.

To confirm the accumulation of unprocessed invertase, and to determine the site of cleavage of the mutant protein, we performed sequential Edman degradation of $^{35}SO_4^{2-}$-[^{3}H]leucine double-labeled proteins. Wild-type forms of invertase accumulated in *sec18*, or secreted in a wild-type strain, were removed from SDS gels of samples that had been treated with endo H. Table II shows that ^{35}S was released in the second cycle of Edman degradation and ^{3}H was released in cycle 11. This was expected from the location of met and leu in mature invertase at position 2 and 11, respectively. s1 invertase that accumulated in a wild-type cell in the apparently unprocessed and processed forms were compared with the mutant secreted form by the same analysis. As expected from the sequence of the gene, ^{35}S was released from the unprocessed form in cycle 1, and ^{3}H in cycles 2, 3, 7, 9, and 10. In contrast to wild-type

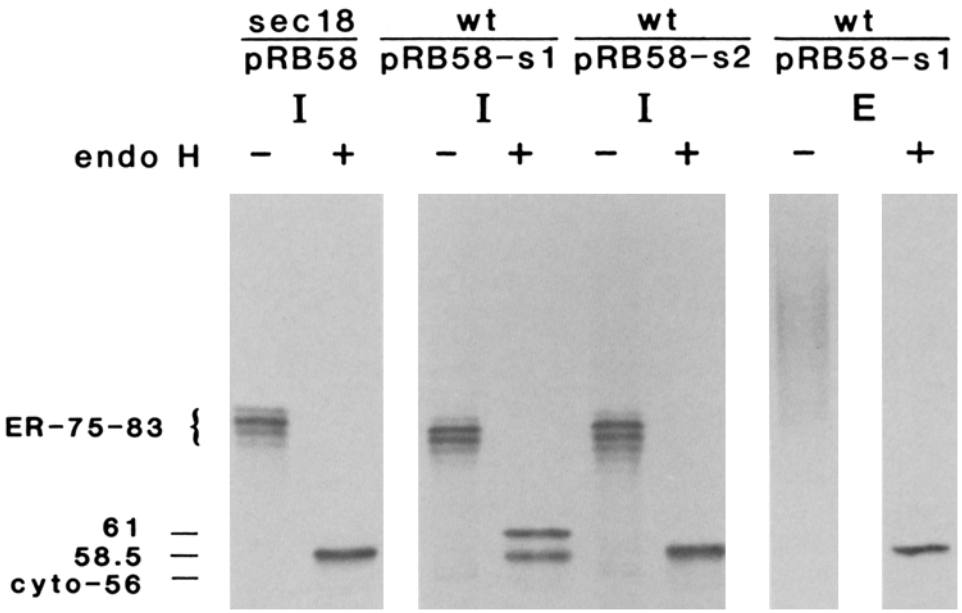


FIGURE 10 Endo H treatment of wild-type and mutant invertases. SEY5188 (*sec18*) transformed with the *SUC2* plasmid and SEY2102 transformed with mutant plasmids were derepressed for invertase expression and labeled with $^{35}SO_4^{2-}$ for 30 min (37°C for SEY5188 and 25°C for SEY2102). A portion of the SEY2102 culture was incubated an additional 2 h under conditions of chase. Spheroplasts formed from each sample were sedimented, and invertase immunoprecipitated from the pellet (*I*) and supernatant (*E*) fractions as before. Samples of each were treated with endo H and compared with untreated material by SDS gel electrophoresis and autoradiography.

TABLE II

s1 Signal Cleavage Occurs at an Alternate Site

		$^{35}SO_4$ in cycle	[^{3}H]leu in cycle
SUC2	ER	2	11
	Secreted	2	ND
SUC2-s1	ER-unprocessed	1	2, 3, 7, 9, 10
	ER-processed	1	10
	Secreted	1	ND

1 2 3 7 9 10

met-leu-leu-gln-ala-phe-leu-phe-leu-leu-ala-gly-phe-ala-ala-lys-ile-

2/1 11/10

ser-(ala / val)-ser-met-thr-asn-glu-thr-ser-asp-arg-pro-leu

* ‡

ND, not determined.
* Normal cleavage site.
‡ s1 mutant cleavage site.

processed invertase, ^{35}S was released in cycle 1 of the processed mutant form, and ^3H in cycle 10 of the accumulated form. Hence, the val-ser bond was not cleaved in s1 mutant invertase; instead, the adjacent ser-met bond was cleaved at a reduced rate.

DISCUSSION

Lesions in a transport signal could, by means of genetic suppressor analysis, lead to the definition of interacting proteins that mediate transport. As a first step in this approach, we have designed a screening procedure to detect mutations in the invertase structural gene that specifically affect secretion. By insisting on retention of full enzyme activity, we should avoid mutations that simply result in denatured protein precipitated in the lumen of a secretory organelle. As an illustration of this point, of 29 independent *suc2* mutant isolates that produce enzymatically inactive invertase cross-reacting material, six were found to accumulate in a core-glycosylated form. These mutations most likely yield denatured invertase that remains in the ER.

Starting with extensively mutagenized *SUC2* plasmid DNA, only three out of 6,000 transformants were found that accumulate active, stable invertase. Of these, two contain the same mutation. The distinct mutations, *SUC2*-s1 and -s2, produce fully active forms of invertase which are delayed in transport from the ER (or possibly some post-ER compartment) to the Golgi body, but not to a similar extent in subsequent export to the cell surface. This delay is considerably greater for s1 than s2.

Signal peptide cleavage in the s1 mutant invertase is deficient owing to the substitution of val for ala at position −1 of the cleavage site. Retention of the signal peptide on about half of the s1 molecules causes invertase to form irregular assemblies, as detected by electrophoresis in a nondenaturing gel system. These assemblies remain associated with ER membranes permeabilized by a weak detergent, saponin, while being readily solubilized by Triton X-100. The other half of the accumulated s1 invertase, which has a polypeptide size characteristic of the proteolytically processed form, may be retained by normal subunit association with the unprocessed subunits. Alternatively, as this material is processed at the adjacent peptide bond, met-terminated invertase may be inherently transport defective.

The mutant invertase is cleaved at a reduced rate at the adjacent ser-met bond. This alternate site has, according to von Heijne's rules (49), roughly equal probability of cleavage in the normal invertase signal peptide. Substitution of val at the normal site increases the probability of cleavage at the alternate site. Additional substitutions at the ser site may further reduce processing and secretion of invertase so that cells would be unable to grow on sucrose or raffinose as a carbon source. Such a situation could be used to obtain extragenic suppressor mutations among which signal peptidase mutants with altered substrate specificity may be found.

It is not clear why the failure to remove a signal peptide has such a dramatic effect on invertase transport from the

ER. The reduced solubility and increased heterogeneity of accumulated s1 invertase suggests that unprocessed enzyme either associates with a stable ER component or aggregates to a point where proper packaging for transport is hindered. In either case, the accumulation has no effect on transport of acid phosphatase, or cell surface growth, so it is unlikely that mutant invertase associates with and titrates some essential ER component.

Other similar effects of signal peptide cleavage inhibition have been noted. Haguenauer-Tsapis and Hinnen (50) have deleted the cleavage site region of the yeast acid phosphatase gene (*PHO5*) and find unprocessed, core-glycosylated enzyme accumulated within the cell which is only slowly secreted. Hortin and Boime (51, 52) report that incorporation of the threonine analogue, β-hydroxynorvaline, at the preprolactin signal cleavage site blocks proper processing. Precursors accumulate in the ER in the unprocessed and in an alternatively processed form. This material is unstable and only slowly secreted in small amounts into the medium. Solubility and membrane association of the unprocessed preprolactin and acid phosphatase have not been investigated; however, failure to cleave the signal peptide of a mutant form of bacterial β-lactamase results in association of the precursor with the external surface of the cytoplasmic membrane (53).

There are many examples of proteins with uncleaved signal peptides, such as yeast α-factor (54), which nevertheless are transported rapidly from the ER. What then is the function of signal peptide cleavage? Wickner (55) has suggested that folding of membrane proteins and perhaps secretory proteins is influenced by the signal peptide. If so, signal peptide cleavage early in the synthesis of a precursor could ensure correct folding. This appears not to be the case for s1 mutant invertase and cleavage-deficient acid phosphatase (50); both are fully active enzymes. Perhaps certain signal peptides are cleaved to prevent association with a stable component of the ER. In this regard, some proteins are designed to remain in the ER, and may retain the signal peptide as a specific anchoring segment.

s2 mutant invertase shows slow transport from the ER with no delay in signal peptide cleavage. With regard to enzyme activity, electrophoretic mobility, ease of release from the ER, and temperature stability, s2 mutant invertase resembles the normal enzyme accumulated in the ER in the pleiotropic mutant *sec18*. Although the mutation results in a hydrophobic substitution (thr → ile at position +64), no effect on subunit interaction or solubility in the presence or absence of detergent was detected. The effect on transport could be due to a subtle conformational change that influences mutant enzyme concentration in a transitional zone of the ER, or to defective interaction with a receptor that mediates invertase movement. It is unlikely that the transport delay results from some nonspecific interaction between s2 mutant invertase and a membrane component because no block occurs in transit from the Golgi body to the cell surface.

If the s2 mutation interferes with an ER transport signal, the interaction between mutant invertase and a receptor may be only partly defective, or the slower rate of transport may reflect a basal level of receptor-independent flow of soluble proteins to the Golgi body. These possibilities would be distinguished by the isolation of additional *SUC2* mutations outside the signal peptide region that further slow the rate of transport.

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